Exhibit A

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Characterization of Echinocandin-Resistant Mutants of *Candida albicans*: Genetic, Biochemical, and Virulence Studies

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The pneumocandins are potent antifungal agents of the echinocandin class which are under development for use as broad spectrum antimycotic therapy. One important consideration for any new therapeutic class for treating serious fungal infections is the potential for drug resistance development. In this study we have isolated and characterized four independent spontaneous *Candida albicans* mutants resistant to the potent semisynthetic pneumocandin L-733,560. These mutants have many of the properties of *FKS1/ETG1* echinocandin-resistant mutants of *Saccharomyces cerevisiae*, including (i) cross-resistance to other 1,3-β-D-glucan synthase inhibitors, such as papulacandin and echinocandins, but no change in sensitivity to other antifungal agents; (ii) in vitro glucan synthase activity that is more resistant to pneumocandins than the wild-type parent enzyme; and (iii) semidominant drug resistance in spheroplast fusion strains. The mutants were compared with *C. albicans* echinocandin-resistant mutants isolated by mutagenesis by L. Beckford and D. Kerridge (mutant M-2) (abstr. PS3.11, *in* Proceedings of the XI Congress of the International Society for Human and Animal Mycology, Montreal, Canada, 1992) and by A. Cassone, R. E. Mason, and D. Kerridge (mutant CA-2) (Sabouraudia 19:97–110, 1981). All of the strains had resistant enzyme activity in vitro. M-2 grew poorly and had low levels of enzyme activity. In contrast, CA-2 and the spontaneous mutants grew as well as the parents and had normal levels of glucan synthase activity. These results suggest that these resistant mutants may have alterations in glucan synthase. CA-2 was unable to form germ tubes, an ability retained by the spontaneous mutants. The virulence of the spontaneous mutants was unimpaired in a mouse model of disseminated candidiasis, while M-2 and CA-2 were 2 orders of magnitude less virulent than their parent strains. Significantly, mice challenged with the spontaneous mutant CAI4R1 responded therapeutically to lower levels of L-733,560 than would be predicted by the increase in in vitro susceptibility.

The pneumocandin and echinocandin lipopeptides are potent antifungal agents which inhibit the synthesis of 1,3-β-D-glucan, an essential fungal cell wall component. The lack of a mammalian cell counterpart suggests that therapeutic agents which inhibit this synthetic process would be free of mechanism-based toxicity. Compounds in this class are now under intensive study for development as broad-spectrum antifungal therapy because of the recent demonstration of efficacy in animal models for aspergillosis (2, 3, 7, 16, 62) and pneumocystis pneumonia (49, 50) as well as for candidiasis (4, 22). One important consideration for any new therapeutic class for treating serious fungal infections is the potential for drug resistance development. Although microbial drug resistance has not been as prevalent for antifungal agents as for antibacterial agents, there have been clinical failures associated with drug-resistant organisms (44, 47). The mechanisms and frequency of resistance emergence depend on the antifungal compound and the target organism. The natural diploidy of *Candida albicans* and its lack of a sexual cycle require that drug resistance mutations be either dominant or present in both alleles for a recessive trait. For example, flucytosine (5FC) is a powerful agent for the treatment of candidiasis, but its use is often limited by the rapid emergence of resistance in a sensitive strain during treatment. In this case, the mechanism of resistance development is understood (14, 56–58). Clinical isolates of *C. albicans* are naturally heterozygous for a variety of recessive mutations, including 5FC resistance (59). It is postulated that under the selective pressure of drug exposure, mitotic recombination yields the homozygous recessive resistance marker. With the increasing use of fluconazole as maintenance therapy for AIDS patients and for prophylaxis in many immunocompromised patient populations, infections with intrinsically resistant species or haploid organisms, such as *Candida krusei* and *Candida glabrata*, respectively, are becoming more common (46, 53, 61). Fluconazole-resistant strains of *C. albicans* have also been isolated (47). At least three mechanisms of fluconazole resistance have been demonstrated, including reduced drug uptake, drug resistance in the target enzyme(s) (lanosterol demethylase and $\Delta^{5,6}$sterol desaturase), and increased lanosterol demethylase activity (reviewed in references 26, 42, 44, and 54).

No clinical data are yet available for resistance emergence for any glucan synthase inhibitors. However, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* mutants resistant to aculeacin A, papulacandin B, and pneumocandins have been isolated in vitro for genetic and biochemical studies and suggest that more than one mechanism can produce lipopeptide resistance in haploid organisms (11, 15, 19, 20, 45). A spontaneous pneumocandin-resistant mutant of *S. cerevisiae* which is 30-fold more resistant than the wild type shows in vitro enzyme activity that is 50-fold less sensitive to the inhibitor than the wild-type enzyme (19). Genetic studies showed that resistant enzyme activity cosegregates with whole-cell resistance and that a mutation in a single gene (*FKS1/ETG1* [echinocandin target gene]) is responsible for both phenotypes. In diploids

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TABLE 1. Strains used in this study

Strain	Origin	Phenotype and/or genotype	Source or reference
6406		Prototroph	L. Beckford
M-2		Ech^r	L. Beckford
MY1055		Wild type used in animal models	1
3153		Wild type	ATCC^a
CA-2		Ech^r	10
CAI4		*ura3*	21
CAI4(Ura$^+$)	pJAM15 (*URA3*)	Ura$^+$	This work
CAI4R1		*ura3* Ech^r	This work
CAI4R1(Ura$^+$)	pJAM15 (*URA3*)	Ura$^+$ Ech^r	This work
1006		*arg57 ser57 lys1 ura3 MPA1*	23
981		*arg57 ser57 lys1 MPA1*	23
hOG839		*ade2 pro mal/+*	R. Poulter
M-2Arg$^-$		*arg57 MPA1* Ech^r	This work
Fusants			
TF2-2, TF2-3, TF2-7, TF2-8, TF2-12	CAI4R1 + 981	*arg57/+ ser57/+ lys1/+ ura3/+ MPA1/+* Ech^r/+	This work
CF2-2, CF2-3	CAI4 + 981	*arg57/+ ser57/+ lys1/+ ura3/+ MPA1/+*	This work
TF1-1, TF1-3, TF1-4	CAI4R1 + hOG839	*ade2/+ pro/+ arg57/+ ura3/+* Ech^r/+	This work
CF1-1, CF1-3	CAI4 + hOG839	*ade2/+ pro/+ arg57/+ ura3/+*	This work
EF1-1, EF1-2, EF1-3	M-2 + 1006	*arg57/+ ser57/+ lys1/+ ura3/+ MPA1/+* Ech^r/+	This work
WTF1-1, WTF1-2, WTF1-3	6406 + 1006	*arg57/+ ser57/+ lys1/+ ura3/+ MPA1/+*	This work
ADF1-7	M-2Arg$^-$ + hOG839	*ade2/+ pro/+ arg57/+ MPA1/+* Ech^r/+	This work

^{a} ATCC, American Type Culture Collection.

the resistance phenotype is semidominant; i.e., diploids heterozygous for the resistance allele (*etg1-1*) have MICs intermediate between those of the wild-type and resistant parents. The mutant is cross-resistant to dihydropapulacandin, aculeacin A, and other pneumocandin analogs but is unaltered in its sensitivity to a large panel of antifungal compounds. Mutant MS10 also showed similar properties (20). The accumulating genetic and biochemical data for *S. cerevisiae* indicate that *FKS1* specifies the catalytic subunit of 1,3-β-D-glucan synthase required for vegetative growth (18, 19, 27, 28). The *FKS2* gene product, with a predicted 90% amino acid identity to Fks1p, can substitute, albeit incompletely, when *FKS1* function is lost by mutation or deletion (18, 36).

Three studies on the emergence of lipopeptide resistance in *C. albicans* have been reported. Mehta et al. described UV-induced mutants resistant to aculeacin A which have alterations in cellular lipids (38, 39). No measurements of glucan synthase activity or drug permeability were presented. Cassone et al. isolated an echinocandin B-resistant mutant of *C. albicans* (CA-2) which has the unusual property of maintaining the yeast form under the in vitro conditions that induce hyphal growth (10). While this strain was not virulent in a mouse model of disseminated candidiasis, it was unexpectedly virulent in a model of murine vaginitis. In the latter model, CA-2 did form pseudohyphal filaments (13). More recently, Beckford and Kerridge reported the isolation of a number of mutagen-induced *C. albicans* and *C. glabrata* mutants resistant to echinocandin B and to the related compound cilofungin (6). One of these strains (M-2) shares properties of the *S. cerevisiae etg1-1* and *etg1-3* mutants with respect to enzymic resistance and semidominance (17). M-2 was also less virulent in a mouse model of disseminated candidiasis (17, 31).

Strains M-2 and CA-2 were derived from mutagenized cultures (3, 6). Several shortcomings of these strains, including alterations in morphology or growth rate and a lack of convenient genetic markers, led us to isolate new mutants of a genetically marked strain, CAI4 (*ura3*) (21). In this paper, we describe the isolation and characterization of four spontaneous *C. albicans* mutants resistant to the potent semisynthetic pneumocandin analog L-733,560. The properties of these mutants were compared with those of M-2 and CA-2. The spontaneous and induced mutants have many of the phenotypes of *S. cerevisiae FKS1/ETG1* echinocandin-resistant (Ech^r) mutants, including specific resistance to 1,3-β-D-glucan synthase inhibitors and resistant enzyme activity. The virulence of the spontaneous mutants was unimpaired in a mouse model of disseminated candidiasis, but infections with the mutants could be treated with L-733,560 at drug doses lower than would have been predicted by in vitro susceptibility testing. The significance of these findings for clinical resistance is discussed.

MATERIALS AND METHODS

Antifungal compounds. Pneumocandin B_0, a naturally occurring pneumocandin (51), and L-733,560 (9), a more potent semisynthetic, water-soluble derivative, were provided by scientists at Merck Research Laboratories, Rahway, N.J. All compounds were shown by high-performance liquid chromatography to be >95% pure. L-687,781 (55) and echinocandin B were prepared at Merck. Fluconazole was obtained from Pfizer Central Research, Groton, Conn.; 5FC was obtained from Hoffmann-La Roche, Nutley, N.J., ketoconazole and itraconazole were obtained from Janssen Pharmaceuticals, Piscataway, N.J.; and terbinafine was obtained from Sandoz Pharmaceuticals, East Hanover, N.J. Aculeacin A (41) was from Toyo Jozo. Amphotericin B, nystatin, and tunicamycin were purchased from Sigma (St. Louis, Mo.).

Strains, media, and growth conditions. The *C. albicans* strains used in these experiments are listed in Table 1. The echinocandin-resistant strain M-2 and its wild-type parent, 6406, were kindly provided by L. Beckford, and strain CA-2 was from A. Cassone. The genetically marked strains 1006 and hOG839 were provided by S. Scherer and R. Poulter, respectively. Cultures were routinely grown at 30°C in yeast extract-peptone-dextrose (YPD) with 100 μg of adenine per ml (YPDA), in Sabouraud dextrose agar, or in synthetic dextrose medium (SD) with the necessary supplements (52). For growth of *ura3* strains, uridine was added to YPDA at 100 μg/ml (YPDAUd). Mycophenolic acid (MPA) was used at 5 to 10 μg/ml in SD where indicated. Ura$^-$ auxotrophs were selected on medium containing 1 mg of 5-fluoroorotic acid (FOA) per ml (8, 21). Solid media contained 1.5% agar. Growth curves were obtained with YPDAUd at 30°C in a Gyrotory water bath (New Brunswick Scientific, Edison, N.J.). Hyphal growth was induced at 37°C in RPMI 1640 medium (Gibco) lacking sodium bicarbonate, with L-glutamine, and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) at pH 7.0. Where indicated, fetal calf serum was added to 10%.

Mutant isolation. Approximately 2×10^7 unmutagenized stationary-phase cells were spread on YPDAUd plates containing 0.2 μg of L-733,560 per ml and incubated at 30°C for 24 to 48 h. Three colonies were purified from a heavy background of residual growth. The number of spontaneous mutations per generation was determined by a fluctuation test as follows. A stationary-phase culture of strain CAI4 was diluted in YPDAUd to give ca. 1 CFU/ml, and 0.5-ml aliquots were grown at 30°C until the tubes with 1 CFU reached stationary phase. Ten cultures were plated onto YPDAUd containing 0.8 μg of L-733,560 per ml, and the number of resistant colonies was scored after 2 days. The mutation rate was calculated from the number of cultures with no resistant colonies and the number of generations from a single colony by the formula $a = (-\ln p_0)/N$ where a is the number of spontaneous mutations per generation, p_0 is the fraction of cultures which had no resistant clones, and N is the number of cell divisions that occurred from a single cell.

Spheroplast fusion and heat shock. Spheroplasts of exponentially growing cells were prepared by enzymic digestion as described previously (32) except that Zymolyase 100T (0.03 μg/ml) was used instead of snail gut enzyme. Spheroplasts were washed with 1 M sorbitol and fused by mixing complementary strains in a fusogen mixture consisting of 20% polyethylene glycol in 10 mM $CaCl_2$–10 mM Tris HCl, pH 7.5. When MPA resistance was used as the selectable marker, the ratio of spheroplasts from strain 1006 (MPA^r) to spheroplasts from the wild type (MPA^s) was 5:1. For strains with complementary auxotrophies, equal numbers of spheroplasts were combined. After 30 min at 30°C, fusion strains (fusants) were harvested, washed with 1 M sorbitol and spread onto selective medium (SD or SD plus MPA). Fusants were purified on selective media twice before characterization. Heat shock (90 s at 51°C) was used to reduce the ploidy of fusants (25).

Antifungal susceptibility testing. MICs and minimum fungicidal concentrations were determined in Yeast Nitrogen Base (Difco) with 1% glucose by the broth microdilution assay described previously (4). Briefly, 10^4 yeast cells were inoculated into 0.150 ml of medium containing twofold serial dilutions of the test compound in microtiter dishes. Growth was monitored visually after incubation for 48 h at 30°C. The MIC was defined as the lowest concentration of drug showing no visible growth. For determinations of minimum fungicidal concentrations, aliquots taken from the microtiter dishes after 24 h of incubation were inoculated onto solidified Sabouraud dextrose agar or SD with uridine and/or adenine added as required. The minimum fungicidal concentration (MFC) was defined as the lowest concentration of drug which reduced the CFU by $\geq 10^3$. Agar diffusion assays on Yeast Nitrogen Base-glucose medium were also used to estimate drug susceptibility. Exponentially growing cultures were inoculated to a density of approximately 10^4 CFU/ml in molten medium cooled to 50°C, and 5-mm-diameter paper disks saturated with the test compound were applied to solidified medium. In some experiments, 10 μl of drug solution was dropped directly onto the solidified agar medium.

Membrane preparation and 1,3-β-D-glucan synthase assay. Glucan synthase activity was measured in *C. albicans* membranes as described previously (19). The assay measures the formation of radiolabeled trichloracetic acid-precipitable material formed from [^{3}H]UDP-glucose. The 1,3-β-D-glucan synthase 50% inhibitory concentration (IC_{50}) was defined as the concentration at which the compound inhibits formation of 50% of the trichloroacetic acid-precipitable polysaccharide. Specific activity is expressed as nanomoles of product formed per hour per milligram of protein.

In vivo virulence test and recovery of yeast cells. Overnight cultures of CA-2, M-2, and their derivatives were grown in SD with necessary supplements or selective agents, washed twice in sterile phosphate-buffered saline, and resuspended in 1/10 of the original culture volume. Because several strains formed unusual morphological forms and clumped together, CFUs did not give an accurate estimate of inoculum size. We found that optical density as a measure of cell mass correlated with hemacytometer counts for all strains. Therefore, the culture density was determined in three ways: hemocytometer count, CFU on SDA (Sabouraud dextrose agar)-uridine plates, and A_{600}. CAI4 and its derivatives were transformed to uridine prototrophy with an integrative vector containing the *C. albicans URA3* gene (34) before virulence was determined, since *ura3* mutants are not virulent in animal models (24, 29). For M-2 and CA-2, five outbred CD-1 mice (Charles River, Wilmington, Mass.) weighing 19 to 21 g were used per group. Complement component C'5-deficient DBA/2N female mice (19 to 21 g) (Taconic Farms, Germantown, N.Y.) were used to determine the virulence of CAI4 derivatives. Mice were injected intravenously in their lateral tail veins with 0.2 ml of serial 10-fold-diluted cell suspensions. The final inocula ranged from 10^4 to 10^7 cells per mouse. One mouse per treatment group was sacrificed at 2, 7, 14, 21, and 28 days after infection. The kidneys were removed and homogenized, and CFU were determined as described previously (4). Morbidity and mortality were recorded daily for 28 days. The 50% lethal dose was calculated by the Knudsen-Curtis method (30). Mutants were tested for susceptibility to L-733,560 or amphotericin B treatment in a disseminated-candidiasis target organ assay described previously (1). In brief, DBA/2N mice were infected with one-half the lethal dose of particular strain. Therapy was initiated intraperitoneally 15 to 30 min after challenge, and mice were treated for a total of 4 days. Efficacy was quantitated by CFU per gram of kidneys at 7 days after challenge. Five mice were used per group per experiment. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meets or exceeds all applicable local, national, and international laws and regulations.

RESULTS

Isolation of spontaneous *C. albicans* mutants resistant to L-733,560. In preliminary studies, the concentration of L-733,560 that inhibited growth of CAI4 on YPDAUd plates was 0.05 to 0.1 μg/ml. Therefore, the first attempt to isolate spontaneous resistant strains was made by using the selective agent at 0.2 μg/ml. Three resistant colonies from 2×10^7 unmutagenized cells were isolated from a single stationary-phase culture and characterized further. The resistance phenotype for each strain was stable in the absence of drug in multiple serial transfers. We have designated the mutants strains CAI4R1, -R2, and -R3. Each strain grew well on YPDAUd plates containing 16 μg of L-733,560 per ml. Since the mutants were isolated from a single culture, we cannot rule out the possibility that they are clonally related, and only CAI4R1 was studied in detail.

Because of high background growth at 0.2 μg of L-733,560 per ml, further tests of spontaneous-mutation frequency were done at 0.8 μg/ml. In three separate trials, the spontaneous-mutation frequency of a bulk culture was between 0.1 and 1 in 10^7/ml. The number of spontaneous mutations per generation as assessed by a fluctuation test was 2×10^{-8} mutations per cell division. Three additional independent mutants, NR2, NR3, and NR4, were isolated in these experiments.

Growth rates and morphologies of resistant mutants. In view of the observation that the echinocandin-resistant mutant CA-2 is unable to undergo the yeast-to-hyphal transition under standard laboratory conditions (10, 13), we examined the growth rates and the filament-forming abilities of the spontaneous resistant mutants. CAI4R1, NR2, NR3, and NR4 grew as budding yeasts in YPDAUd medium. The mutants were able to germinate and produce normal hyphal filaments with the same kinetics and efficiency as the parent in RPMI 1640 at 37°C (data not shown). The growth rate of each spontaneous mutant was indistinguishable from that of the wild-type parent (data not shown). In contrast, M-2 grew significantly more slowly than its parent, 6406, with a doubling time of 130 min in YPD at 30°C compared with 71 min for 6406 (Fig. 1). This growth defect was more pronounced at 37°C, where the doubling time for strain 6406 was 53 min compared with 130 min for M-2. In addition, M-2 formed unusual morphological forms in YPD liquid and solid media, with many enlarged cells which formed clumps that were difficult to dissociate with mechanical mixing. M-2 did not form hyphae in YPD with 10% fetal calf serum, while 6406 produced a few hyphal filaments under these conditions (data not shown). Neither 6406 nor M-2 produced detectable hyphae in RPMI 1640 with 10% fetal calf serum, but M-2 showed many abnormal forms, some resembling multicellular pseudohyphal growth. CA-2 yeast cells did not germinate under any conditions.

Specificity of drug resistance to glucan synthesis inhibitors. The susceptibilities of the strains to a wide variety of antifungal agents with different mechanisms of action and different chemical structures were measured in an agar diffusion assay. Results with selected compounds for CAI4R1 and its wild-type parent are shown in Table 2. CAI4R1 is resistant to the chemically related compounds aculeacin, echinocandin B, and pneumocandins, which are known to inhibit glucan synthesis, and partially resistant to dihydropapulacandin (L-687,781), a structurally distinct inhibitor of this enzyme activity. The mutant was not resistant to a wide variety of antifungal antibiotics

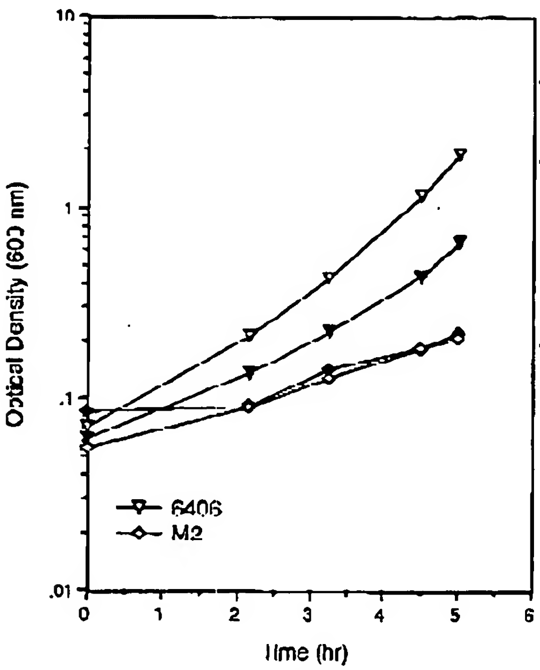

FIG. 1. Growth of *C. albicans* 6406 and M-2 (Ech[r]) in liquid YPD. Saturated cultures of each strain at 30°C were subcultured into fresh medium to an initial optical density at 600 nm of ca. 0.1. The cultures were grown with shaking at 30°C (closed symbols) or 37°C (open symbols). Optical densities of samples were measured at the indicated times.

(data not shown), including agents in clinical use, such as amphotericin B, itraconazole, flucytosine, and fluconazole (Table 2). M-2 showed a similar pattern of specificity except that it was not resistant to dihydrepapulacandin (data not shown).

Sensitivity of glucan synthase to pneumocandin is reduced in mutant strains. In view of the specificity of the drug resistance of the mutants, the glucan synthase activities in *C. albicans* membranes prepared from the echinocandin-resistant mutants CA-2, M-2, and CAI4R1 were characterized with respect to specific activity and inhibition by the pneumocandin L-733,560. Table 3 shows that M-2 had only 9% of the glucan synthase activity measured in crude extracts of strain 6406. This activity was at least 5,000-fold less sensitive to inhibition by L-733,560 than that in the wild type (IC_{50}s of >20 and 0.004 μM, respectively). The spontaneous mutant CAI4R1 was different from M-2 in that the apparent IC_{50} for L-733,560 inhibition was only fourfold greater than that of its parent (Table 3). However, inspection of the inhibition curves (Fig. 2) reveals that glucan synthase activity from CAI4 can be inhibited more than 90% by L-733,560 at 20 μM, but the inhibitory effect on the enzyme from CAI4R1 does not exceed 55%, even at the highest concentrations of the drug. The enzyme from CA-2 was completely resistant to drug concentrations as high as 20 μM.

Semidominance of echinocandin resistance in spheroplast fusion strains. In *C. albicans*, dominance of a resistance marker can be assessed by fusing spheroplasts of diploid strains to create tetraploids and/or polyploids that are maintained by selective pressure. Assuming that a resistant strain is heterozygous for the mutant trait and the fusion partner is homozygous for the wild-type phenotype (sensitivity), the resulting tetraploid will be resistant for a fully dominant marker. With a semidominant trait, one resistance allele in a tetraploid could

TABLE 2. Susceptibilities of CAI4 and CAI4R1 to antifungal antibiotics

Compound	μg/spot	Zone of inhibition (mm) with:	
		CAI4	CAI4R1
Cell wall active			
Aculeacin A	5	26	11
Echinocandin B	20	28	9
Pneumocandin B_0	20	39	10
L-667,781	10	16	11
Tunicamycin	10	18	19
Clinical			
Fluconazole	5	23 (h[a])	26
Ketoconazole	10	35 (h)	33
Itraconazole	10	24	23
Amphotericin B	4	19	19
Nystatin	10	10	10
Flucytosine	1	24	31

[a] h, hazy zone.

TABLE 3. L-733,560 inhibition of whole cells and glucan synthase activity from echinocandin-resistant mutants

Strain	Sp act[a]	MIC (μg/ml)	IC_{50} (μM)
6406	17	0.36	0.004
M-2	1.5	25	>20
CA-2	16	>32	>30
CAI4	46	0.125	0.2
CAI4R1	55	32	0.8

[a] Expressed as nanomoles · hour^{-1} · milligram of protein^{-1}.

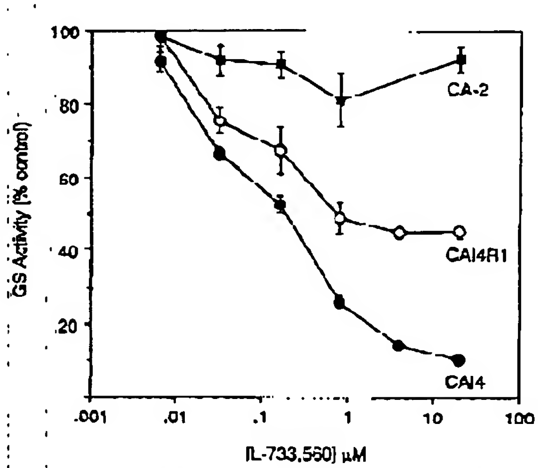

FIG. 2. Inhibition of glucan synthase (GS) activity by L-733,560. Crude membranes from CAI4, CAI4R1, and CA-2 were prepared and assayed for GS activity as described in Materials and Methods. L-733,560 in water was added to reaction mixtures at final concentrations of 0.0064 to 20 μM. The amount of product synthesized without L-733,560 represents 100% for each GS preparation. Bars indicate standard deviations.

TABLE 4. Sensitivities of M-2 fusion strains to L-733,560

Strain composition and/or name	MIC (μg/ml)
1006 + M-2	
EF1-1	1.5
EF1-2	3.0
EF1-3	0.36
1006 + 6406	
WTF1-1	0.36
WTF1-2	0.36
WTF1-3	0.36
1006	0.36
M-2	25
6406	0.36
M-2Arg⁻ + hOG839	
ADF1-1	1.5
ADF1-2	1.5
ADF1-3	1.5
ADF1-4	1.5
ADF1-5	1.5
ADF1-6	1.5
ADF1-7	6.25
M-2Arg⁻	12.5
hOG839	0.06

confer partial resistance, or it may not be sufficient for phenotypic expression of resistance, such that the mutation will appear to be recessive in a tetraploid but not in a diploid. With these considerations in mind, M-2 was fused with 1006, a multiply auxotrophic strain which carries the dominant selective marker for MPA resistance (*MPA1*). Selection for fusion was based on prototrophy and MPA resistance. Individual fusants from this procedure are designated EF1-1, -2, and -3. The unselected marker, echinocandin resistance, was determined for isolated fusants by using unsupplemented MPA-containing medium. Fusants constructed from the wild-type parent and 1006 served as controls (strains WTF1-1, -2, and -3). Quantitative MIC results for EF1-1 and EF1-2 showed increased echinocandin resistance compared with that of control fusants WTF1-1, -2, and -3 (Table 4). Because this method cannot guarantee that fusants are fully tetraploid, the lack of dominance in the third strain (EF1-3) may be due to loss of the chromosome which carries the resistance allele. Conversely, resistance in EF1-1 and EF1-2 could be the result of chromosomal loss of the wild-type allele. We sought to distinguish between these alternatives by analyzing segregants derived from the fusants produced by heat shock, a procedure which causes a reduction of tetraploids to diploidy and aneuploidy. Survival after heat shock varied between 1 and 20%, and survivors were tested for echinocandin resistance and recovery of auxotrophic markers. None (<0.15%) of the heat shock progeny of EF1-3 were echinocandin resistant segregants, but we did recover L-733,560-resistant strains from EF1-1 and EF1-2 at frequencies of 2 and 0.5%, respectively (data not shown).

In the process of analyzing heat shock progeny from the EF1-1 fusion, a strain carrying the echinocandin resistance allele and an arginine auxotrophic marker was isolated (M-2Arg⁻). The presence of an auxotrophic marker allowed independent verification of the semidominance of resistance with a second fusion. M-2Arg⁻ was fused with hOG839 (*ade2 pro met/+*), and seven of the nine prototrophs isolated were characterized. Six of the seven fusants had intermediate resistance to L-733,560. The remaining fusant was almost as resistant as M-2Arg⁻ (Table 4). These results are consistent with those predicted for a mutation conferring dominant or semidominant resistance in diploid and tetraploid strains.

A similar assessment of dominance was conducted for CAI4R1 (Ura⁻) by fusing it with strain hOG839 and selecting for prototrophs. Fusants constructed from the wild-type parent and hOG839 served as controls. Three CAI4R1 fusants (TF1-1, TF1-3, and TF1-4) and two CAI4 fusants (CF1-1 and CF1-3) were analyzed for echinocandin resistance on plates. TF1-1 and TF1-4 failed to grow on L-733,560-containing plates; TF1-3 grew well. Both CAI4 fusants were pneumocandin sensitive. Quantitative liquid MIC data confirmed the results observed on plates (Table 5). The resistant fusant (TF1-3) and a sensitive fusant (TF1-1) were subjected to heat shock, and the stable *ura3* marker was selected directly by spreading heat-shocked cultures onto FOA plates. The uridine-requiring isolates were scored for unselected auxotrophic markers and L-733,560 resistance. Survival after heat shock was high (60 to 80%), and few auxotrophs were recovered. All of the tested FOA-resistant segregants from TF1-3 (24 of 24) were resistant to L-733,560. However, only 11 of 24 FOA-resistant segregants from TF1-1 were resistant to pneumocandin. Each of the 11 Ech^r Ura⁻ strains was Ade⁻, as would be expected for two markers on the same chromosome (35). The single Pro⁻ segregant we recovered was L-733,560 resistant.

To test the idea that the mutation in CAI4R1 is semidominant in diploids but recessive in tetraploids, a second fusion was performed with the multiply auxotrophic strain 981 to produce strains TF2-1 through TF2-13. Fusants constructed from the wild-type parent and 981 served as controls (CF2-1 through CF2-13). Nine of 13 TF2 fusants grew on medium containing 0.8 μg of L-733,560 per ml, but none of the 13 CF2 fusants grew at the same drug concentration. Representative results from a liquid MIC assay are shown in Table 5. Four L-733,560-resistant TF2 fusants (TF2-2, TF2-3, TF2-8, and TF2-12) and one sensitive fusant (TF2-7) were heat shocked, and the segregants were analyzed as described above. Two CF2

TABLE 5. Sensitivities of CAI4R1 fusion strains to L-733,560 and recovery of resistant segregants after heat shock

Strain composition and/or name	MIC (μg/ml)	% Resistant strains after heat shock (% of Ura⁻)
hOG839	0.06	
CAI4R1	32	
CAI4	0.125	
981	0.5–2	
CAI4R1 + hOG 839		
TF1-1	0.125	46
TF1-3	8.0	100
TF1-4	0.25	
CAI4 + hOG 839		
CF1-1	0.125	
CF1-3	0.125	
CAI4R1 + 981		
TF2-2	2–16	100
TF2-3	R[a]	100
TF2-7	1.0	31
TF2-8	8–32	12
TF2-12	2–8	36
CAI4 + 981		
CF2-2	0.25	<6
CF2-3	0.06	<6

[a] R, resistant on plates with 0.8 μg of L-733,560 per ml; the MIC in liquid was not determined.

TABLE 6. Fifty percent lethal doses (LD_{50}s) for *C. albicans* strains in a disseminated candidiasis model

Strain	LD_{50} (CFU/mouse [10^4]) on day:		
	7	14	21
CAI4(Ura⁺)	1.3	0.53	0.53
CAI4R-1(Ura⁺)	0.39	0.17	0.17
3153	3.7	3.7	ND[a]
CA-2	100	67	ND

[a] ND, not determined.

fusants served as controls. Two of the resistant fusants (TF2-2 and TF2-3) were refractory to heat shock killing, as all of the input cells survived the standard protocol and all of the segregants were FOA resistant, Ura⁻, and L-733,560 resistant. The remaining strains (TF2-7, TF2-8, and TF2-12) were sensitive to heat shock, with survival values between 40 to 70%. Each fusant gave rise to fully L-733,560-resistant colonies after heat shock when uridine prototrophs were selected on FOA. In two separate experiments with TF2-7, 1 of 40 and 15 of 48 FOA-resistant colonies were fully resistant to L-733,560. Three lysine and three arginine auxotrophs were recovered. Similar results were obtained for TF2-8 and TF2-12 (2 of 16 and 9 of 25 L-733,560-resistant strains among FOA-selected colonies, respectively). The control fusions gave the frequencies typical of Ura⁻ auxotrophs after heat shock, and none of the 37 FOA-resistant colonies we recovered was resistant to L-733,560.

Virulence studies. The virulence of the echinocandin-resistant mutant M-2 was at least 30-fold reduced compared with that of its parent strain in a survival study using CD-1 mice (50% lethal doses at 14 days postinfection of 1.7×10^7 and 5×10^5 CFU per mouse, respectively). Cultures grown from isolated colonies recovered from infected kidneys retained their resistance to L-733,560. Strain CA-2 and Ura⁻ derivatives of CAI4R1 and CAI4 were tested for virulence in DBA/2N mice, a more sensitive model for disseminated candidiasis. The results from a representative trial (Table 6) demonstrate that the spontaneous CAI4R1 mutant was as virulent as CAI4(Ura⁺) in this animal model. In contrast, and in accord with previous reports (13), CA-2 was at least 1 order of magnitude less virulent than the parent strain 3153. Interestingly, as many as 10^5 CFU per g of kidney were recovered from the survivors of CA-2-infected mice inoculated with 10^5 cells.

The full virulence of CAI4R1(Ura⁺) in animal models allowed us to test whether in vitro resistance was manifest as resistance to drug treatment in vivo. Despite an increase in the MFC of L-733,560 of more than 1,000 fold (from ≤0.06 to 64 μg/ml), the disseminated candidiasis produced by CAI4R1 (Ura⁺) was still susceptible to treatment with L-733,560, and the 99% effective dose in the disseminated-candidiasis model was increased only 8-fold (Table 7). Similar results were obtained for the spontaneous Echr^r mutants CAI4R2(Ura⁺) and CAI4R3(Ura⁺), strains which may be clonally related to CAI4R1(Ura⁺) (data not shown). Infections with each of the strains were equally sensitive to amphotericin B treatment.

TABLE 7. In vitro and in vivo susceptibilities of mutant and wild-type strains to L-733,560[a]

Strain	MFC (μg/ml)		ED_{99} (mg/kg)	
	AMB	L-733,560	AMB	L-733,560
CAI4(Ura⁺)	0.25	≤0.06	0.06	0.05
CAI4R1(Ura⁺)	0.125	64	0.03	0.40

[a] MFC, minimum fungicidal concentration; ED_{99}, 99% effective dose; AMB, amphotericin B.

DISCUSSION

The *C. albicans* echinocandin-resistant mutants analyzed in this work have several properties in common with analogous semidominant echinocandin-resistant mutants (R560-1C, MS10, and MS14) of *S. cerevisiae* (19, 20). First, the resistance phenotype of the mutants is specific to inhibitors of glucan synthesis. Susceptibility to inhibitors with other modes of action was unaffected. All of the mutations conferred at least a 10-fold-increased resistance to lipopeptide inhibitors of glucan synthase (Table 3 and unpublished data). Multidrug resistance mechanisms such as drug efflux do not seem to contribute to the echinocandin resistance phenotype. From a clinical perspective, it is encouraging that M-2 and CAI4R1 are still susceptible to clinically relevant therapies, i.e., amphotericin B, fluconazole, itraconazole, and 5FC, in vitro (Table 2 and unpublished data). Moreover, all of the mutants are sensitive to amphotericin B in a murine model of candidiasis (Table 7 and data not shown). In addition, an amphotericin B-resistant *C. albicans* strain is still susceptible to echinocandin in vitro (12).

Growth under laboratory conditions is unimpaired for five of the six L-733,560-resistant *Candida* mutants and several of the *S. cerevisiae etg1* Ech^r mutants. The specific activity of 1,3-β-D-glucan synthase from these strains is equivalent to that of the wild type, and therefore, effects on growth would not be expected. Strain M-2 grows quite slowly, but its growth impairment may not be due entirely to the Ech^r phenotype. M-2Arg⁻, the segregant isolated from the fusion of hOG839 and M-2, was as resistant to L-733,560 as its parent, but this isolate had faster, albeit still impaired, growth (unpublished data). Further analysis of M-2Arg⁻ will be required to establish the role of the echinocandin resistance mutation in the growth rate in this strain. Despite the difference in growth rate, M-2Arg⁻ and M-2 are nearly equally echinocandin resistant in vitro (Table 4), which eliminates slow growth as the primary reason for drug resistance.

The most significant similarity between the *C. albicans* echinocandin-resistant mutants and the *S. cerevisiae* echinocandin-resistant mutants is the diminished susceptibility to inhibition by pneumocandins of the in vitro glucan synthase activity. The glucan synthase activities from CA-2 and M-2 were the most resistant of the *Candida* mutants, with IC_{50}s of >20 μM, an increase of more than 5,000-fold compared with the wild type. As in the case of the *S. cerevisiae etg1-1* mutant (19), the greater-than-50-fold increase in MIC for CA-2 and M-2 can be explained by a qualitatively large increase in the IC_{50} for the in vitro enzyme. The results for CAI4R1 are more difficult to explain by the same model. The MIC for this strain is increased at least 100-fold, while the IC_{50} is increased only 4-fold. The fact that wild-type enzyme activity is inhibited by 90% with 20 μM L-733,560 but the mutant enzyme cannot be inhibited to an equivalent extent, even at 32 μM L-733,560 (data not shown), suggests that the inhibition curve represents a mixture of sensitive and resistant enzyme activities (Fig. 2). This would be expected if the mutants are heterozygous with respect to the resistance locus and the mutation is semidominant in diploids. Growth at high concentrations of L-733,560 would be dependent on the residual 45% of glucan synthase activity detected in vitro. *S. cerevisiae* mutants with an insertion-deletion at the *FKS1* locus have approximately 20% of wild-type in vitro glucan synthase activity; presumably, the remaining glucan synthase activity is from the redundant, but alternatively regulated, *FKS2* gene (18, 36). Al-

though such strains grow more slowly than the wild type, they are able to survive. The MIC assay used to determine echinocandin susceptibility is an endpoint assay and may not be able to detect the subtle differences in the growth rates with and without the drug. We have demonstrated that for *C. albicans*, the MIC of L-733,560 correlates with the concentration of drug that inhibits cell wall glucan synthesis in whole cells by 80% as measured by incorporation of radiolabeled glucose in polymers (33, 43).

Because of the limitations of the methods, the genetic analysis of the *C. albicans* mutants presented here supports, but can not prove, that the mutations we describe are dominant or semidominant. Parasexual analysis requires the formation of tetraploids by cell fusion and subsequent reduction in ploidy by heat shock. Using this method, we obtained fusants (EF1-1 and EF1-2) of M-2 and 1006 that clearly behaved as if they were tetraploid for the chromosome that carries the resistance allele. Echinocandin resistance was intermediate in such fusants. As expected, fully resistant strains were recovered among the progeny produced by heat shock of EF1-1 or EF1-2. Fusant EF1-3 was the exception to this pattern because it did not yield resistant segregants after heat shock. We believe that this fusant was not tetraploid for the resistance locus and did not carry the Ech^r allele. Efforts to select fusants of strain CA-2 with strain 1006 proved problematic because of significant growth by CA-2 and its parent on MPA plates (unpublished data).

The fusants of CAI4R1 to hOG839 or 981 presented a more complicated pattern because of the difficulties of hybrid analysis in *C. albicans*. Even when nuclear fusion has been achieved, each hybrid formed by protoplast fusion can have a different genome stability (48). Although there is a formal possibility that prototrophs obtained by fusion may be heterokaryons, fusants selected for MPA resistance should be mononuclear, since the resistance marker is not dominant in heterokaryons (23). However, strains TF1-3, TF2-2, and TF2-3 must have been unstable nuclear fusants, because growth on nonselective media produced uniform colonies which were all phenotypically like CAI4R1, i.e., fully resistant to L-733,560 and Ura^-. Two classes of true fusants were obtained. The behavior of the first class (TF1-1 and TF2-7) suggests that the mutant allele from CAI4R1 must be recessive; the fusants were fully sensitive to the pneumocandin, but resistant segregants were recovered after heat shock (Table 5). In contrast, the behavior of the second class (TF2-8 and TF2-12) implies that the mutation is semidominant; the strains had intermediate resistance, and resistant segregants were recovered after heat shock. One explanation for these results is that fusions in the first class may be fully tetraploid for the resistance locus (Ech^r/Ech^s/Ech^s/Ech^s) and that one mutant allele is insufficient to markedly alter sensitivity to the drug in whole cell assays. Because the selection procedure used only four genetic markers, this analysis can not guarantee that all chromosomes are tetraploid in any individual fusant. Fusants in the second class may be polyploid, but they may be diploid or aneuploid at the resistance locus (Ech^r/Ech^s or Ech^r/Ech^r/Ech^s), yielding resistance levels closer to the original parent. There are intrinsic differences in the echinocandin susceptibilities of the various wild type strains used for spheroplast fusions. Drug sensitivity would therefore depend upon which "wild-type" Ech^s allele the aneuploid strain retains. Work with recessive and dominant 5FC resistance demonstrated that the most common origin of recessive segregants derived from tetraploid hybrids was a reduction of ploidy rather than recombination (59, 60). We have recently used a cloned fragment of the *C. albicans FKS1* homolog to create targeted disruptions in CAI4R1. Our results suggest that the mutant has one copy of a wild-type *FKS1* homolog and one copy of a resistance allele (31, 40).

The level of L-733,560 resistance of strain CAI4R1 (Ura^-) in MFC assays (1,000-fold) was not commensurate with the increase in ED_{99} in the animal model for disseminated candidiasis (Table 7). Further work is needed to define the relationship between in vitro susceptibility results and in vivo activity results for this class of compounds. McIntyre and Galgiani (37) have shown that the in vitro susceptibilities of *C. albicans* strains to several antifungal antibiotics are dependent on the growth medium. In their study, the in vivo efficacy of cilofungin, an echinocandin B analog, correlated more closely to the in vitro susceptibility at pH 3.0 than at pH 7.4 (37). It will be important to develop correlations of in vitro susceptibility, in vivo activity in animal models, and clinical outcome when the echinocandin class of antifungal agents are tested in clinical trials. If laboratory-generated mutants such as CAI4R1 are predictive of the type of pneumocandin resistance mutations that may arise in vivo, we anticipate that such mutated strains may not pose a significant clinical problem.

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